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Antifungal Compounds in the Ethyl Acetate Soluble Fraction of the Extractives of Taiwania *(Taiwania cryptomerioides* Hayata*)* Heartwood

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Keywords

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Summary

This study was to isolate and identify the antifungal compounds in the ethyl acetate soluble fraction of the methanol extractives of Taiwania (*Taiwania cryptomerioides* Hayata) heartwood and to examine their antifungal activity. Five compounds were obtained by open column chromatography and HPLC and based upon the results from Mass, ^{1}H-NMR, and ^{13}C-NMR analyses. Their structures were identified, namely ferruginol, helioxanthin, savinin, taiwanin C, and hinokiol. According to the results of antifungal test, the order of antifungal index of these compounds for *Coriolus versicolor* (L. ex Fr.) Quel. was ferruginol > taiwanin C > savinin > hinokiol. For *Laetiporus sulphureus* (B. ex Fr.) Bond. it was taiwanin C > savinin > ferruginol > hinokiol.

Introduction

Taiwania (*Taiwania cryptomerioides* Hayata) is an economically important tree species indigenous to Taiwan. The heartwood of Taiwania is yellowish red with distinguished purplish pink streaks. With regard to the decay resistance, Taiwania is classified into the excellent durability species in Taiwan. It is well known that extractives influence the durability of wood. There were many kinds of extractives isolated and identified from Taiwania, including eleven lignans, eight flavonoids, twenty one sesquiterpenoids, eighteen diterpenoids, three lipids, two cyclitols, and one steroid that summarized in the Wang *et al*.'s review paper (Wang *et al.* 1997, 1998; Su *et al*. 1998). However, there has been few research investigating the relationship between the wood properties and the extractives of Taiwania. Recently, we have tried to illustrate the chemical constituents that contribute to the color of Taiwania heartwood and their mechanisms of structure conversion. One of the color substances, taiwanin A, was isolated from Taiwania heartwood. According to the ^{13}C-NMR, ^{1}H-NMR, HSQC, HMBC, and NOE difference spectroscopy analyses, the diene structure of taiwanin A was reconfirmed to be the *trans-trans* formulation. In addition, it was proven that deep orange crystalline taiwanin A changed to white and pale yellow compounds, taiwanin C and taiwanin E after light irradiation (Chang *et al*. 1999). Besides that, the relationship between extractives and decay resistance of Taiwania was also discussed in our previous paper. Five compounds, including taiwanin A, α-cadinol, α-cedrol, hinokiol, and sugiol, were isolated from hexane soluble fraction of the methanol extractives of Taiwania heartwood. Based on the results of antifungal test, the order of antifungal index of these compounds for *Coriolus versicolor* (L. ex Fr.) Quel. was α-cadinol > α-cedrol > hinokiol > sugiol > taiwanin A. For *Laetiporus sulphureus* (B. ex Fr.) Bond., it was α-cadinol > hinokiol > α-cedrol > taiwanin A. Among these, α-cadinol showed the best antifungal effectiveness. It completely inhibited the growth of *C. versicolor* and *L. sulphureus* at 100 ppm (Chang *et al.* 1998). In addition to hexane soluble fraction of the methanol extractives of Taiwania heartwood, the ethyl acetate soluble fraction also showed the antifungal effectiveness. This study is, moreover, tried to isolate and identify the antifungal compounds in the ethyl acetate soluble fraction of the methanol extractives of Taiwania heartwood and to examine their antifungal activity.

Material and Methods

General

HPLC was performed with a Jasco model PU980 pump equipped with a Jasco UV970 UV detector and the column used was a Hibar Lichrosorb Si 60 (25 × 1 cm i.d.). The IR spectra were recorded on a Bio-rad model FTS-40 spectrophotometer. The MS was obtained on Finnigan MAT-958 Mass spectrometer. The NMR spectra were recorded on a Bruker Avance-500 MHz FT-NMR.

Extraction and isolation

Twenty seven aged Taiwania used in this study was collected from the Experimental Forest of National Taiwan University. Taiwania heartwood chips were prepared from a green cut tree. The air dried chips (5.7 kg) were exhaustively extracted with methanol (MeOH). The extractives were condensed to *ca* 286.4 g, then extracted with *n*-hexane (*n*-C_6H_{14}), chloroform ($CHCl_3$), ethyl acetate (EtOAc), and methanol (MeOH), successively. After removing solvents from

the combined extractives, the *n*-C_6H_{14}, $CHCl_3$, EtOAc, and MeOH soluble fractions and MeOH insoluble fraction were obtained. Followed by chromatographing with silica-gel column eluted with EtOAc/*n*-C_6H_{14} (grading from 0/100 to 100/0), EtOAc soluble fraction (5 g) was divided into 13 subfractions (E1-E13). Compound **1** was isolated and purified from E3 by semi-preparative HPLC (Mobil phase: EtOAc/*n*-C_6H_{14} = 30/70; Flow rate: 6 ml/min; Retention time (R.t.) = 2.4 min). E5 subfraction, additionally, was isolated to give compound **2** (R.t. = 4.8 min), compound **3** (R.t. = 5.4 min), compound **4** (R.t. = 7.6 min), and compound **5** (R.t. = 8.7 min) by the same HPLC system (Mobil phase: EtOAc/ *n*-C_6H_{14} = 35/65; Flow rate: 6 ml/min).

Antifungal assays

Fungi used in this study were *Coriolus versicolor* (L. ex Fr.) Quel. and *Laetiporus sulphureus* (B. ex Fr.) Bond.. All antifungal assays were performed three times, and data were averaged. All subfractions (E1 to E13) and compounds (compound **1** to compound **5** as shown in Fig. 1) that isolated from E3 and E5 were added to sterilized potato dextrose agar (PDA) to give 100 ppm concentrations of extractives. After transferring the mycelium of *C.v.* and *L. s.*, the testing plates were incubated at 27 °C when the mycelium of fungi reached the edge of control plate (without adding extractives), the antifungal index was calculated as follow:

Antifungal index = (1- Da/Db) 100

Da: radial growth mycelium on the experimental plate
Db: the diameter of testing plate.

*Compound **1***

Yellow oil; EIMS for $C_{20}H_{30}O$ found 286; IR ν max : 3370, 1612, 1579, 1501 cm^{-1}; ^{1}H-NMR (in $CDCl_3$) : δ (ppm) 0.89 (3H, s, H-18), 0.93 (3H, s, H-19), 1.15 (3H, s, H-20),1.22 (3H, d, *J* = 7.0 Hz, H-16), 1.29 (3H, d, *J* = 7.0 Hz, H-17), 2.77 (1H, m, H-7a), 2.81(1H, m, H-7b), 3.11 (sept, *J* = 7.0 Hz, H-15), 4.81 (s, OH), 6.62 (1H, s, H-11), 6.81 (1H, s, H-14).

*Compound **2***

Yellow crystal; m.p.: 240–241 °C; EIMS for $C_{20}H_{12}O_6$ found 348; UV : λ_{max}^{MeOH}: 262 and 290 nm; ^{1}H-NMR (in $CDCl_3$) : δ (ppm) 5.15, 5.23 (each 1H, d, *J* = 15.0 Hz, H-9'), 5.94, 5.95 (each 1H, d, *J* = 1.5 Hz, 3'-O-C-O-4'), 6.03, 6.06 (each 1H, d, *J* = 1.4 Hz, 4-O-C-O-5), 6.77 (1H, dd, *J* = 7.7 Hz, *J* = 1.6 Hz, H-6'), 6.79 (1H, d, *J* = 1.6 Hz, H-2'), 6.87 (1H, d, *J* = 7.7 Hz, H-5'), 7.30 (1H, d, *J* = 8.7, H-3), 7.68 (1H, d, *J* = 8.7, H-2), 8.40 (1H, s, H-7).

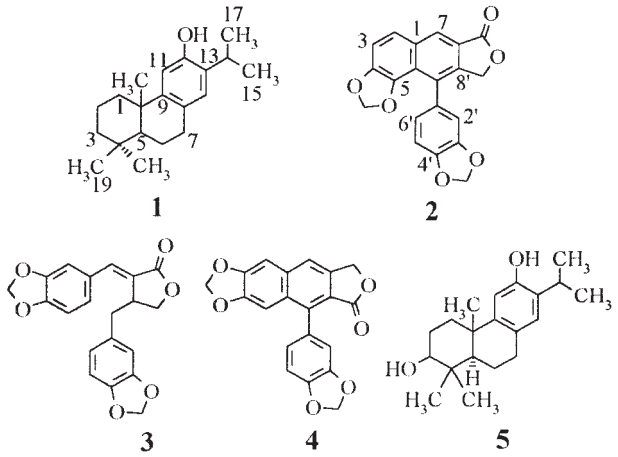

Fig. 1. Configuration of five compounds from the ethyl acetate soluble fraction of Taiwania heartwood (**1**) ferruginol, (**2**) helioxanthin, (**3**) savinin, (**4**) taiwanin C, (**5**) hinokiol.

*Compound **3***

Yellow crystal; m.p. : 146–147 °C; EIMS for $C_{20}H_{16}O_6$ found 352; UV : λ_{max}^{MeOH} : 240, 295 nm; IR ν max : 1730, 1640, 1595, 1500, 1255, 1180, 1030, 920, 825, 800 cm^{-1}; ^{1}H-NMR (in $CDCl_3$) : δ (ppm) 2.56 (dd, *J* = 14.2, 4.2 Hz, H-7'a), 2.97 (dd, *J* = 14.1, 9.7 Hz, H-7'b), 3.71 (m, H-8'), 4.24 (m, H-9'), 5.90 5.92 (each 1H, d, *J* = 1.3 Hz, 3'-O-C-O-4'), 6.61 (dd, *J* = 8.0, 1.2 Hz, H-6), 6.71 (d, *J* = 8.0 Hz, H-5'), 6.85 (d, *J* = 8.0 Hz, H-5), 7.02 (d, *J* = 1.4 Hz, H-2), 7.06 (dd, *J* = 8.0, 1.4 Hz, H-6'), 7.48 (s, H-7).

*Compound **4***

White solids; m.p. : 267–270 °C; EIMS for $C_{20}H_{12}O_6$ found 348; UV : λ_{max}^{MeOH}: 258, 294, 350 nm; IR ν max : 3030, 1760, 1620, 1500, 930, 800 cm^{-1}; ^{1}H-NMR (in $CDCl_3$) : δ (ppm) 5.35 (2H, s, H-9), 6.03 (2H, s, 3-O-C-O-4), 6.06 (2H, s, 3'-O-C-O-4'), 6.77 (d, *J* = 7.8 Hz, H-6'), 6.78 (s, H-2'), 6.93 (d, *J* = 7.8 Hz, H-5'), 7.09 (s, H-5), 7.17 (s, H-2), 7.66 (s, H-7).

*Compound **5***

White crystal; m.p. : 233–234 °C; EIMS for $C_{20}H_{30}O_2$ found 302; IR ν max : 3353, 1607, 1578, 1504 cm^{-1}; ^{1}H-NMR (in $CDCl_3$) : δ (ppm) 0.87 (3H, s, H-19), 1.04 (3H, s, H-18), 1.16 (3H, s, H-20),1.19 (3H, d, *J* = 7.0 Hz, H-16), 1.22 (3H, d, *J* = 7.0 Hz, H-17), 2.75 (1H, m, H-7a), 2.85 (1H, m, H-7b), 3.08 (sept, *J* = 7.0 Hz, H-15), 4.47 (s, OH), 6.59 (1H, s, H-11), 6.82 (1H, s, H-14).

Results and Discussion

There are no doubts that the most significant factor influencing the durability of wood are extractives. To obtain the antifungal extractives in Taiwania, we extracted the Taiwania heartwood with MeOH, completely. The MeOH extractives were extracted with *n*-hexane (*n*-C_6H_{14}), chloroform ($CHCl_3$), ethyl acetate (EtOAc), and methanol (MeOH). After removing solvents from the combined extractives, the *n*-C_6H_{14}, $CHCl_3$, EtOAc, and MeOH soluble fractions and MeOH insoluble fraction were obtained. According to our previous study (Chang *et al.* 1998), both *n*-C_6H_{14} and EtOAc soluble fractions present the antifungal effectiveness. We have also demonstrated that α-cadinol isolated from *n*-C_6H_{14} soluble fraction has strong antifungal ability. In this study, we continually analyzed the antifungal compounds in the EtOAc soluble fraction of Taiwania heartwood extractives.

EtOAc soluble fraction was divided to thirteen subfractions (E1 to E13) with open column chromatograph. Figure 2 presents the antifungal index of E1-E13 subfractions from the EtOAc soluble fraction. It was very obvious that, compared with other subfractions, E3 (the antifungal index is 100.0 for *Laetiporus sulphureus* (B. ex Fr.) Bond.) had excellent antifungal ability and E5 (the antifungal index is 52.4 for *Coriolus versicolor* (L. ex Fr.) Quel.) also had very good antifungal ability. Therefore, we isolated the E3 and E5, moreover, with HPLC.

When E3 was analyzed by HPLC (Mobil phase: EtOAc/*n*-C_6H_{14} = 30/70; Flow rate: 6 ml/min), we found that the peak appeared at 2.4 min (Fig. 3), was the major compound of E3. After isolation and purification by the semi-preparative HPLC (use the same isolated condition), the compound **1** was obtained. According to the results obtained from ^{1}H-NMR, mass spectrum, compound **1** was confirmed to be ferruginol, whose structure was elucidated

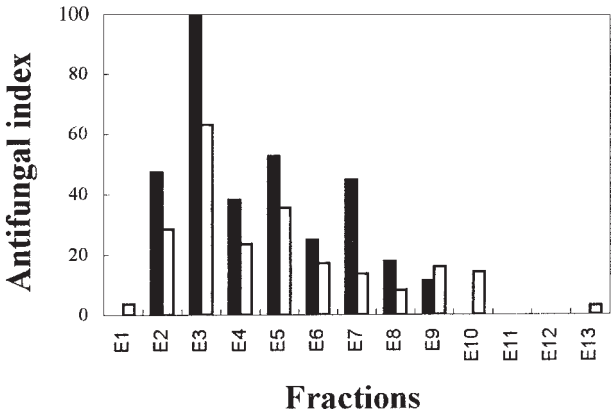

Fig. 2. Antifungal index of E1-E13 fractions from the ethyl acetate solution of Taiwania heartwood extractives. ■ L. s., □ C. v.

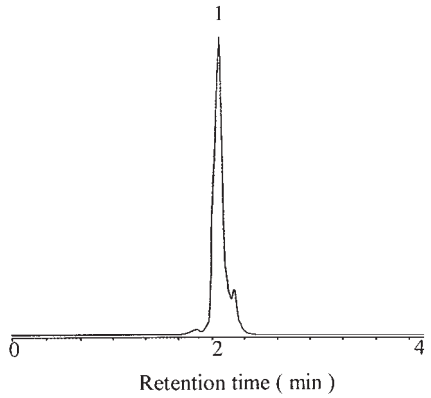

Fig. 3. HPLC chromatogram of E-3 fraction.

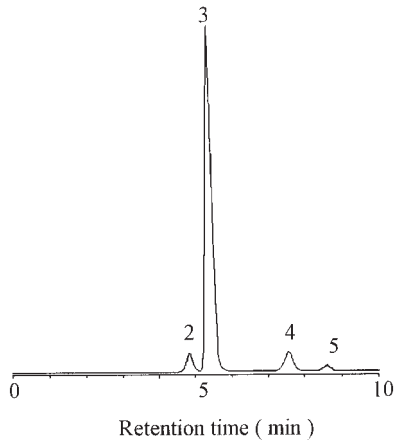

Fig. 4. HPLC chromatogram of E-5 fraction.

by Lin and coworkers and isolated from the bark of *Libocedrus formosana* (Lin *et al.* 1975). Ferruginol isolated from *Podocarpus* spp. has also been demonstrated to be an antifungal compound (Rudman 1965) against *Lentinus lepideus*. According to our experimental results (Table 1), its antifungal index for *C. versicolor* was 50.1 and for *L. sulphureus* was 51.3 at 100 µg/ml.

Table 1. Antifungal index of five compounds from the ethyl acetate soluble fraction of the methanol extractives of Taiwania heartwood (at 100 µg/ml)

Compounds	Fungi	Antifungal index
Ferruginol	*C. v.*	50.1
	L. s.	51.3
Helioxanthin	*C. v.*	26.0
	L. s.	36.1
Savinin	*C. v.*	38.8
	L. s.	56.0
Taiwanin C	*C. v.*	48.6
	L. s.	66.3
Hinokiol	*C. v.*	26.2
	L. s.	27.5

L. s.: *Laetiporus sulphureus*; *C. v.*: *Coriolus versicolor*.

At the same time, E5-subfraction was also further isolated by HPLC. The mobile phase used to isolate E5 was EtOAc/*n*-C_6H_{14} = 35/65 and flow rate was 6 ml/min. Figure 4 is the HPLC chromatogram of E5-subfraction. It was found that there were four compounds in the E5-subfraction. After isolation and purification with the same HPLC system, compound **2** (R.t. = 4.8 min), compound **3** (R.t. = 5.4 min), compound **4** (R.t. = 7.6 min), and compound **5** (R.t. = 8.7 min) were obtained. M.p., MS, and ^{1}H-NMR spectra were used to identify these compounds. Compound **2** is helioxanthin that is one of the chemical constituents contributing to the heartwood color of Taiwania (Wang *et al.* 1998). In addition, componds **3** and **4** are savinin and taiwanin C that have been isolated from Taiwania heartwood by Su *et al.* (1998). Compound **5** is hinokiol, moreover, that was also isolated from *n*-C_6H_{14} soluble fraction in our previous paper (Chang *et al.* 1998).

The antifungal index of five compounds against *C. versicolor* and *L. sulphureus* was presented in Table 1. Based on the results of antifungal test, the order of antifungal index of these compounds for *C. versicolor* was ferruginol > taiwanin C > savinin > hinokiol. For *L. sulphureus* it was taiwanin C > savinin > ferruginol > hinokiol.

Conclusion

To elaborate methods to prolong the life time of wood for human is the duty of wood utilization researchers. There are two reasonable ways to approach that. One is to choose the high durability species, another is to treat the wood with preservatives, such as CCA, CCB...and so on, or with chemical modifications. Although wood protection with preservatives is an effective method to prolong the life time of wood products, it may pollute the environment. As regard to the environmental protection, to find the bioactive constituents in the durability species and to elucidate the mechanisms of them are the best ways to achieve wood protection without polluting the environment in the next century. Taiwania is an endemic tree that grows on elevations from 1800 to 2600 m of central mountains in Taiwan. With regard to the decay resistance, Taiwania is classified into the excellent durability species in Taiwan. According to our serial

researches, several antifungal compounds, such as α-cadinol, α-cedrol, hinokiol, sugiol, taiwanin A, ferruginol, helioxanthin, savinin, and taiwanin C, were isolated from the heartwood of Taiwania. Among these, α-cadinol, ferruginol, and taiwanin C were demonstrated to possess the most antifungal effectiveness. We will try to illustrate if there are synergistic effects among these compounds? And how they can defend the wood against the fungi? The study will be under investigation.

References

Chang, S.-T., C.-L. Wu, S.-Y. Wang and Y.-C. Su. 1998. Studies on the antifungal compounds in the heartwood extractives of Taiwania (*Taiwania cryptomerioides* Hayata) (I): Isolation and identification of antifungal compounds in hexane soluble fraction. For. Prod. Industries. *17* (2), 287–304.

Chang, S.-T., S.-Y. Wang, Y.-C. Su and Y.-H. Kuo. 1999. Chemical constituents and mechanisms of discoloration of Taiwania (*Taiwania cryptomerioides* Hayata) heartwood—(I) The structure reconfirmation and conversion mechanism of Taiwanin A. Holzforschung *53*, 142–146.

Lin, Y.-T., Y.-H. Kuo and B.-H. Chang 1975. Studies on the extractive constituents of the bark of *Libocedrus formosana* florin. II. J. Chinese Chem. Soc. *22*, 331–334.

Rudman, P. 1965. The causes of natural durability in timber. XVIII. Further notes on the fungi toxicity of wood extractives. Holzforschung *19*, 57–58.

Su, Y.-C., S.-T. Chang, S.-Y. Wang, C.-L. Wu, S.-L. Huang and Y.-H. Kuo. 1998. Structure elucidation of three lignans isolated from Taiwania (*Taiwania cryptomerioides* Hayata) heartwood: Savinin, Taiwanin C, and Dimethylmatairesinol. Q. J. Chin. For. *31* (3), 287–295.

Wang, S.-Y., S.-T. Chang, Y.-C. Su and Y.-H. Kuo. 1997. Studies on the extractives of Taiwania (*Taiwania cryptomerioides* Hayata): a review. Quarterly Journal of the Experimental Forestry on National Taiwan University. *11* (4), 67–81.

Wang, S.-Y., S.-T. Chang, C.-L. Wu, Y.-C. Su, S.-L. Huang and Y.-H. Kuo. 1998. Three chemical constituents contributing to the color of Taiwania (*Taiwania cryptomerioides* Hayata) heartwood: Hinokinin, Helioxanthin, and Pluviatolide. Q. J. Chin. For. *31* (2), 187–193.

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